NATIONAL BUREAU OF STANDARDS REPORT

NBS PROJECT
0708-11-07260

December 31, 1962

NBS REPORT
7825

Progress Report
on
Studies of Dental Calculus: Organic Portion

by

Martha D. Peiperl*
John W. Stanford*

* Research Associate, American Dental Association
Research Division, National Bureau of Standards
Washington, D. C.

This work is a part of the dental research program conducted at the National Bureau of Standards in cooperation with the Council on Dental Research of the American Dental Association; the Army Dental Corps; the Dental Sciences Division of the School of Aviation Medicine, USAF, and the Veterans Administration.

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Studies of Dental Calculus: Organic Portion*

Abstract

The organic portion of dental calculus formed *in vivo* has been studied using electrophoretic and spectrophotometic methods. Emphasis was placed on the determination of the type of protein present. The results indicate the presence of a glycoprotein confirming early work by this laboratory. Small amounts of carboxylic acid (probably citric acid) were found in salivary calculus as well as in duct calculus. Preliminary examinations of the organic portion of calculus formed *in vitro* indicated that it is similar to calculus formed *in vivo*.

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* This investigation was supported in part by U.S.P.H.S. research grant D-902 to the American Dental Association from the National Institute of Dental Research, National Institutes of Health.

1. Introduction

Dental calculus accumulates to varying degrees in the mouths of many people. It is regarded by many as one of the primary causes of periodontal diseases. This report is based on a continuation of the work conducted previously by the Dental Research Section on the chemical analysis of various types of dental calculi [1]. The previous work was concerned with the determination of the elemental constituents of the organic portion of dental calculus which amounts to about 13 percent of the deposit. The analyses and preliminary microradiography of the junction of calculus to the tooth tissue suggested that the organic portion of calculus may play a role in both the calcification process and in the adherence of the calculus to the tooth tissue. The presence of a mucopolysaccharide was indicated but not confirmed in the previous report and approximately 20 percent of the organic matrix was lost or undetermined.

2. Materials and Methods

2.1 Glycoprotein

The mucopolysaccharide fraction of salivary calculus was investigated by two different electrophoretic separations, high voltage electrophoresis on filter paper and low voltage electrophoresis on cellulose acetate strips.

The samples examined consisted of both stimulated and unstimulated saliva, mucin precipitated with acetone from stimulated saliva, EDTA demineralized composite salivary calculus, powdered duct calculus, powdered molar tooth, and powdered artificial calculus*.

For high voltage work, 5.0 mg demineralized calculus were dissolved in 10 ml 1 N ammonium hydroxide (the samples did not dissolve completely). Lambda pipettes were used to place 75λ (microliters) of solution as a round spot on Whatman #1 chromatographic paper. The samples were added to the center of 62 cm long by 3.8 cm wide strips. Six such strips were run simultaneously. Several different buffers were tried in an attempt to make the glycoprotein move away from the origin:

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* Received from Dr. F. J. Draus, University of Pittsburgh.

Veronal-acetate buffer pH 8.6 μ=0.1 [2]
Barbital-borate buffer pH 8.6 μ=0.1 [3]
Borate buffer pH 10 [4]
Borate buffer pH 7.5

A water cooled high voltage electrophoresis apparatus constructed at the National Bureau of Standards after a design by D. Gross [5] was used. The samples were run at 1000 volts for from one to six hours. All strips were dried immediately at 110° C for 10 minutes. The proteins were fixed on the paper by dipping in 10 percent mercuric chloride in ethanol. The papers were then dried and the glycoprotein was stained with Schiffs' reagent* after periodic acid oxidation [6].

For the low voltage work, from 20 to 100 mg of sample were dissolved in 5 ml portions of 10 percent citric acid. Mucin, however, was prepared by adding a 10 mg sample to 5 ml of 1 N NH_4OH. The samples were heated to boiling and then placed on a mechanical shaker for two hours (these samples did not dissolve completely).

Lambda pipettes were used to place 20 λ of solution as a line on cellulose acetate strips.** The samples were placed 3 cm from the anode end of 12 by 2.5 cm strips. Eight such strips were run simultaneously using two different buffers as follows:

Barbital-borate buffer pH 8.6 [3]
Barbital buffer pH 8.6 [7]

The instrument used was a Shandon Electrophoresis Apparatus coupled with a Vokam constant voltage/constant current D.C. power supply.

* Obtained from Fisher Scientific Co.

** Oxoid cellulose acetate strips obtained from Consolidated Labs., Inc., Chicago Heights, Illinois.

Groups of eight samples were run at 8 ma constant current for from forty to ninety minutes. The voltage achieved with the barbital-borate buffer was about 75 v and with the barbital buffer about 180 v.

All strips were dried immediately (under tension) at 100° C for 10 minutes. The glycoprotein was stained with Schiffs' reagent* according to the instruction booklet received with the cellulose acetate strips.

A standard glycoprotein, bovine serum fraction VI** was run along with the unknown samples and it proved to be similar to the second glycoprotein band of saliva.

2.2 Total Protein

Total protein was examined in saliva, mucin, duct calculus, artificial calculus, salivary calculus, and whole tooth. The same samples were used as in low voltage electrophoresis of glycoprotein. Twenty lambda samples were run in barbital buffer [7] on cellulose acetate strips for 90 minutes at a constant current of 1 ma per cm strip width. The initial voltage was 185 v.

The strips were stained for total protein by two different methods: 1. with nigrosine which is the most sensitive protein stain; 2. with amido black which is the most quantitative protein stain.

For nigrosine staining the strips were first dried for 10 minutes at 100° C. They were then placed in a bath of 0.02 percent nigrosine dissolved in 5 percent aqueous acetic acid for 2.5 hours, rinsed in distilled water, rinsed in 10 percent acetic acid, and finally rinsed in water again. After blotting and drying under pressure, the cellulose acetate strips displayed well defined blue protein bands on a faint pink background.

* Obtained from Fisher Scientific Co.

** Cal. Corp. for Biol. Research Los Angles, Cal.

For amido black staining [8] 3.0 g amido black* and 25 g zinc sulfate were dissolved in 500 ml of wash reagent. The wash reagent was prepared by diluting 470 ml of reagent grade methanol to 900 ml with distilled water, adding 100 ml glacial acetic acid, and mixing well.

As soon as the voltage was turned off, the wet strips were placed in the bath of amido black. The staining tray was rotated gently by hand for five minutes. The strips were then rinsed three times in the wash reagent described above and twice in absolute methanol. After blotting the strips were dried under pressure at room temperature. Black bands were obtained on a white background.

Total proteins were also determined in stimulated saliva, composite salivary calculus (20 mg in 5 ml 10 percent citric acid) artificial calculus (25 mg in 5 ml 10 percent citric acid), and tooth (50 mg in 5 ml 10 percent citric acid) all in the presence of urea to prevent the proteins from aggregating.

The buffer was prepared by dissolving 2 ml formic acid, 0.4 g NaOH and 480 g urea in a 1 liter beaker full of distilled water on a magnetic stirrer heater.

Ten lambda of saliva and 20 of the other samples were placed in the center of 12 cm cellulose acetate strips which were run in the urea buffer at 1/2 ma/per strip constant current for one hour with an initial voltage of 295 v. The strips were dried for 10 minutes at 100° C and then stained for total protein with nigrosine and for glycoprotein with Schiffs' stain.

2.3 Carboxylic Acids

The large amounts of citric acid present in other calcified tissues prompted a determination of the citric acid content of calculus. The determination was made on samples of powdered molar tooth, salivary duct stone, calculus from dentures, subgingival composite calculus, and paraffin stimulated saliva.

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* Obtained from Fisher Scientific Co.

A qualitative estimation was made using cellulose acetate low voltage electrophoresis. Calibration curves were first run on standard solutions of 0.0152 N citric acid and 0.0169 N aconitic acid, as titrated against standardized carbonate free NaOH. The calibration curves were determined with 1λ, 3λ, 5λ, 7λ, and 10λ samples applied as bands with lambda pipettes and placed 3 cm from the anode end of the cellulose acetate strips.

The buffer described by D. Gross [10] was used, consisting of 57 ml formic acid and 114 ml acetic acid added to 3 liters distilled water and diluted to 4 liters. All samples were run in the Shandon electrophoresis apparatus with a Vokam power supply at a constant current of 1 ma per 2.5 cm strip for 40 minutes. The strips were dried under tension for 10 minutes at 100° C and then placed in the hood under draft for two hours to evaporate off the acetic-formic acid background. An aniline-glucose stain was used to detect the carboxylic acid. The stain was prepared by dissolving 0.5 g glucose in 5 ml distilled water, 0.5 ml aniline, 5 ml 100 percent ethanol and 15 ml n-butanol. The strips were sprayed gently with freshly prepared stain and allowed to air dry. They were then heated at 100° C for 30 minutes to bring out the color of the stain.

After the strips had cooled, a 1 cm wide band containing the brown carboxylic acid band was marked off along with 0.5 cm bands on either side for a blank. These areas were cut out with a razor blade and placed in brown glass vials. The cellulose acetate pieces were dissolved with 3.0 ml of chloroform; ethanol (90:10) solution with some shaking. The solutions were read immediately in a Beckman D. U. using 1 cm wide quartz cells and wavelength of 286mμ (previously determined by running the absorption curve on one sample). The reference cell contained a clean 1 cm band of cellulose acetate dissolved in 3.0 ml of the same chloroform:ethanol solution. Each blank was subtracted from its sample mate to obtain the optical density of the carboxylic acid band.

The precision of this method was such that samples had to be run in octuplicate and the results analyzed statistically. In this manner straight line calibration curves (least square lines) could be obtained with a standard error of the mean of about 6 percent for acontic acid and 4 percent for citric acid. The standard deviations for sets of 8 points were about 20 percent for aconitic and 12 percent for citric acid.

The following samples were run qualitatively by the same method as the citric acid standards but only one sample was run and the bands were not cut out or measured:

1. EDTA demineralized composite calculus in acetic formic acid buffer and in 0.5N NaOH.
2. Whole composite calculus in acetic formic acid buffer, o.1N HCl, 1N HCl, distilled water, 15 percent EDTA, 0.5N NaOH, and a boiling water extract.
3. Test solutions of 15 percent EDTA, H_3PO_4, phosphoric acid-citric acid mixtures with and without $Ca(OH)_2$, 1N HCl.
4. Salivary duct stone in 0.1N HCl and 1N HCl.
5. Crushed whole molar in 0.1N HCl and 1N HCl.
6. Samples dissolved in 30 ml 10 percent trichloroacetic acid + 3 ml H_2SO_4, 1N, 2N, 4N acetic acid, 3N H_3PO_4, 1N HNO_3, 1N H_3PO_4, 10 percent lactic acid.
7. Various combinations of the above samples and various concentrations of samples.

These samples were run in several different buffers and for various lengths of time in an attempt to separate the carboxylic acid band from a solvent band given by HCl and HNO_3.

In the spectrophotometric method of Zipkin [11, 12] for citric acid, a calibration curve was first run on a citric acid standard solution containing 0.1 mg/ml. One ml, 2 ml, 3 ml, 4 ml, and 5 ml samples were pipetted into 50 ml graduated centrifuge tubes and diluted to 20 ml with distilled water. To these were added 0.3 ml 18N H_2SO_4, 0.2 ml 1M KBr, and 0.5 ml saturated $KMnO_4$. The samples were allowed to stand for 15 minutes and then 3 percent H_2O_2 was added dropwise to the disappearance of the brown MnO_2. Excess H_2O_2 was destroyed by adding 0.1N $KMnO_4$ and the samples were made up to 30 ml with distilled water. Thirteen ml n-hexane were added and the centrifuge tubes were covered with a plastic wrap held on by a rubber band.

The tubes were shaken vigorously for about 15 seconds and then centrifuged for 5 minutes at about 1000 rpm. A 10 ml aliquot of supernatant liquid was transferred to another centrifuge tube where 3.0 ml of a 50% dioxane-water mixture and 3.0 ml 4% Na_2S (freshly prepared and filtered) were added to it. The tube was then covered with plastic wrap using a rubber band, shaken vigorously for about 15 seconds, and centrifuged for five minutes at about 1000 rpm.

The solutions were read immediately in a Beckman D. U. in 1 cm quartz cells at 450 mμ [11] and a straight line calibration curve was easily obtained. This calibration curve was used to measure the citric acid content of powdered molar tooth, salivary duct stone, calculus from dentures, composite sub-gingival calculus, and stimulated saliva. Forty mg of sample were dissolved in 15 ml 1N HCl with slight heating. The calculus samples were also placed on a magnetic stirrer heater for six hours in an attempt to dissolve them completely (a few mg residue still remained). The entire sample was filtered into a 50 ml graduated centrifuge tube and washed with 5 ml hot water. The procedure used to obtain the citric acid calibration curve was continued from this point.

Two 15 ml samples of paraffin stimulated saliva were collected in 50 ml glass-stoppered erlenmeyer flasks, each containing 0.5 ml concentrated H_2SO_4. The flasks were shaken and 10 ml aliquots were transferred to test tubes containing 2 ml of 18N H_2SO_4. The tubes were placed in a boiling water bath for 20 minutes, filtered hot, and washed with 5 ml hot water. Five tenths ml saturated bromine water was added and the samples were placed in the refrigerator for two hours. After precipitation of any bromination products the samples were filtered and washed with 5 ml hot water into 50 ml graduated centrifuge tubes and continued as with the citric acid standards.

2.4 Preliminary Study of Artificial Calculus

A sample of artificially grown calculus was received from Dr. F. J. Draus of the University of Pittsburgh and was partially analyzed to determine its similarity to natural salivary calculus.

The calculus was dried in a desiccator over drierite to constant weight. A sample of 0.2071 g dried calculus was placed in cellophane dialysis tubing 1 1/8 inch in diameter and 50 ml 15 percent EDTA (pH 7.0) was added to it. The tubing was tied off with dental floss and suspended in a 400 ml beaker containing 250 ml 15 percent EDTA. The outer solution was stirred with a magnetic stirrer. The outer solution was changed twice and 20 ml aliquots titrated with 0.4 M $CaCl_2$ in the presence of 10 ml 5 percent ammonium oxalate to the first permanent turbidity. The EDTA reached a constant titration value in two changes.

The sample was removed from the dialysis bag, washed with distilled water several times using centrifugation, and dried to constant weight in a desiccator and then in an oven at 100° C.

Samples of whole artificial calculus were also examined for total protein and glycoprotein by the methods already described.

3. Results and Discussion

3.1 Glycoprotein

Two glycoproteins were shown to be present in saliva and mucin by cellulose acetate electrophoresis. One of these glycoproteins travels towards the cathode in a basic buffer (pH 8.6) solution and the other remains at the point of origin. In filter paper electrophoresis these two spots appear as concentric circles around the origin. This glycoprotein which remains at the origin was also found in salivary calculus, duct calculus, artificial calculus, and tooth.

In the presence of a strong urea buffer, saliva and tooth were separated into three glycoprotein bands and salivary calculus and artificial calculus were separated into two glycoprotein bands. In each case one of the bands remained at the origin.

3.2 Total Protein

Stimulated saliva was separated into as many as six bands in barbital-acetate buffer [8] using a nigrosine stain.

Using the method already given in 2.2, mucin was separated into three bands and one diffuse area while duct calculus, artificial calculus, and salivary calculus showed one main band at the origin with some diffuse area. Whole tooth gave three bands and a diffuse area.

In the presence of a strong urea buffer saliva, salivary calculus, artificial calculus, and tooth were all separated into two strong bands. One of these bands remains at the origin while the other band along with some diffuse area travels towards the cathode.

3.3 Carboxylic Acids

Cellulose acetate electrophoresis indicated the presence of a single carboxylic acid in tooth and calculus. This carboxylic acid is most probably citric acid.

The spectrophotometric determination gave the following approximate values of weight percent for citric acid:

Crushed whole molar	0.5%
Duct calculus	0.05%
Calculus from dentures	0.01%
Sub-gingival calculus	0.02%
Stimulated saliva	0.0004%, 0.0006%

The values for tooth [12] and saliva [11] agree with those of Zipkin.

3.4 Artificial Calculus

The organic content of artificial calculus as prepared by demineralization with 15 percent EDTA was 12.7 percent for desiccator dried and 12.4 percent for 100° C dried. This is within the range of salivary calculus found by Stanford and Forziati. [1].

Whole artificial calculus was found to contain only one main protein band which was found to be a glycoprotein when stained with Schiffs' stain. This agrees with the single protein-glycoprotein band found in salivary calculus.

4. Summary

1. There appears in the presence of urea to be two glycoproteins present in salivary and artificial calculus.

2. Tests for total protein in the presence of urea resulted in a minimum of two separate protein bands by electrophoresis of samples of artificial calculus, salivary calculus and human teeth. Since urea is known to cause unfolding of the protein helix, the two bands, and also the two glycoprotein bands, may possibly come from one protein.

3. Salivary calculus as well as duct calculus contains a small amount (0.01 to 0.05%) of carboxylic acid which is probably citric acid.

4. The preliminary examinations of the organic portion of calculus formed *in vitro* indicate that it is similar to calculus formed *in vivo* and bears further study.

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